Transcriptional analysis of the *dachsous*gene uncovers novel isoforms expressed during development

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ABSTRACT

The *Drosophila* cadherin-related protein Dachsous (Ds) plays a prominent role in planar cell polarity (PCP) and growth. The regulation of these two processesis based on the interaction between Ds and Fat proteins, generating an intracellular response required for tissue polarization and modulation of Hippo pathway activity. Here we have performed a comprehensive molecular study of the *ds*gene during larval development that has shown an unexpected complexity in its transcriptional regulation and revealed the expression of hitherto unsuspected transcripts.Also, knockdown of several isoforms provides new evidence on the importance of the cytoplasmic domain in the mechanism of action of Ds during development.

KEYWORDS: Drosophila / growth / PCP/ Dachsous transcripts / DsIntra isoform.

HIGHLIGHTS

- Three novel transcriptional isoforms of *ds*gene are expressed during larval development.
- The cytoplasmic domain of Dsplays an essential role in control growth and PCP.
- Ds regulates the mitochondrial activity.

INTRODUCTION

Numerous tissuesundergo an additional level of organization through an evolutionary conserved mechanism called planar cell polarity (PCP), which contributes to the development of fully functional organs of a precise size and shape. The *Drosophila* cadherin-related Dachsous (Ds) and Fat (Ft) proteins and their homologues in vertebrates, participate in the control of PCP and growth (reviewed in [1]). The current model is based on the heterophilic interaction between these two single-pass transmembrane proteins. This interaction generatesasignaling cascade mediated by the Ft intracellular domain and resulting in cytoskeletal reorganizationand modulation of the Hippo pathway[2, 3].

During Drosophila wing formation, Dsregulates different processes such as the orientation of cell division, specification of proximal-distal (P/D) patterning, vein formation and wing hairs orientation [4-6]. Thus, herein the term PCP/patterning will be used to encompass all these PCP processes.

In addition, Ds can control tissue growth, modulating the activity of Hippo signalling pathway.This role of Ds is related to its ability to regulate the expression of genes such as *four jointed (fj)* [7] and*dally* and *dally-like* (*dlp*),key modulators of the Hegdehog, Wnt and TGF-ß signalling pathways involved in cell proliferation and patterning [8];[9].

Significantprogress has been made in understanding the mechanism by which Ft regulates PCP andgrowth, due to the identification of cytoplasmic partners that interact with specific sequence motifs of Ft cytoplasmic domain (CD) [10-13]. However, less understood is the molecular mechanism of Ds regulating PCP and Hippo pathway.Reported data provide genetic evidencethat Ds can regulate these processes in two different ways: cell-autonomous and non-cell autonomous manner. Therefore,PCPand growth can bealso regulated by Ds independently of Ft [7, 8, 14, 15].At present, it is difficult to envision how the current model, based on the requirement of the extracellular domain of Ds, can explain the cell-autonomous function.

In this work, the molecular analysis of the *ds* gene reveals the expression of several *ds* transcriptional variants in imaginal discs and brain during larval development. We have

characterized three novel isoforms that encode the soluble proteins DsEx, Ds1 and DsIntra. Moreover, we show that the cytoplasmic isoforms can regulate not only PCP/patterning and growth but also mitochondrial activity,indicating a relevantrole of the cytoplasmic domain in the mechanism of action of Ds during development.

MATERIALS AND METHODS

Drosophila genetics

ds mutant alleles ds^{1}, *30AG4*and ds^{38k}, andthe Gal4 lines *638-Gal4*, *en-Gal4,hh-Gal4* and *tub-Gal4,*were obtained from the Bloomington Stock Center. *puc-lacZ* (puc^{E69}*;* [16]. RNAi lines were obtained from VDRC (http://stockcenter.vdrc.at/control/main). $ds^{1}$and *30AG4* are weak alleles caused by transposable elements inserted in coding exons of the extracellular domain [17]. ds^{38k} is a spontaneous mutation not affecting the coding exons (our sequencing analysis). In turn, ds^{36D} is a strong allele generated by P-excision of ds^{05142}[5]. UAS-DsFL[18] comprises the complete coding region of full-length Ds [17]. UAS-ectoDs encodesa large extracellular domain anchored to the cell membrane [15]. The UAS-DsIntraV5 transgene was generated by fusion of the cDNA sequence (nt 10153 to 11464; Genbank: L08811.2) and one copy of V5 epitope to the C-terminus (Ct) in the pUAS-T vector. The UAS-DsExGFPtransgenecomprises from exon2 to exon10 (nt 796 to 6832; Genbank: L08811.2) and one copy of GFP sequence joined to the 3' end of exon10. The aa sequences of the fusion are: UAS-DsIntraV5 …GTRMSRGPFE**GKPIPN…** and UAS-DsExGFP …LITTVGAGT**MVSKGE…** (*ds* sequences are underlined and V5 and GFP sequences appear in boldface).To analyze wing phenotypes, the transgenes *ds*RNAi-cyt, *ds*RNAi-ex, DsFL, UAS-ectoDs, DsExGFP and DsIntraV5 were overexpressed in a wild-type (wt) background under the control of *638G4, enG4*and *hhG4* drivers. For quantitation,the A-PCV and wing area between 4 and 12 wings of each genotype were traced using Adobe Photoshop CS3. The average of distance and area was normalized to control females.Measurement of A-PCV distance was made as described in [11].The wing

area parameter was calculated dividing the posterior area(P)by the total area (T) (Figure S1).

Statistical analysis

In all the experiments, significance was determined using the Student´s t-distribution (two tailed; two sample equal variances). A *p*value of <0.05 was considered statistically significant.

Immunohistochemistry

Wing imaginal discs were dissected and stained as described in [19]. Primary antibodies used for histology were: rabbit anti-Dachsous (Ds^{ex} 1:1000 [20], guinea pig anti-Dachsous (Ds^{cyt} 1:500; this work), mouse anti-COXIV (1:50; Invitrogen), rabbit anti-β-galactosidase (1:10000; Cappel), rabbit anti-Caspase 3 (1:200, Cell Signalling), mouse anti-Dlp (1:100; Developmental Studies Hybridoma Bank)and mouse anti-V5 (1:2000; Invitrogen).Proteins were visualized using fluorescent secondary antibodies (Jackson InmunoResearch Laboratories).Analysis of Ds expression in mitochondriawas determined by co-immunostaining in fat body cells of third instar larvae [21]. ROS production was evaluated in live tissues using Dihydroethidium (DHE), the *Drosophila*imaginal discs were dissected following the protocol described in [22].The guinea pig Ds antibody against the cytoplasmic region was generated from a GST fusion protein (pGEX4T2 vector) containing the amino acids 3410-3700 (AAF51468.3). Imaginal discs were mounted in Vectashield (Vector Laboratories) and Mowiol. Images were acquired using a Zeiss Confocal LSM510 microscope.

Quantitative real-time PCR and data analysis

Total RNA was extracted from three independent biological samples (imaginal discs and brains) of wt and *tubG4/UAS-dsRNAi* larvaeto determine the expression of individual exons by two-step real-time PCR (RT-PCR). The first strand cDNA was synthesized with the

Transcriptor First Strand cDNA Synthesis kit (Roche) using 2 µg of total RNA and oligo(dT) primer. For the second step of qRT-PCR amplification, a pair of primers for each coding exon was designed following the recommendations of the Universal Probe Library Assay Design Center (Roche Applied Science). The *Tbp* and *eIF-2α* genes were used as endogenous controls for analysis of RNA quantity and quality. Primer sequences and amplicon size are indicated in Table S1. All PCR reactions were carried out in technical duplicates using HotStart Taq polymerase (Qiagen) and SYBR green (Qiagen) in an optical 384-well plate with ABI 7900HT (Applied BioSystems). For absolute quantification (standard curve method), the *pUAS-dsFL* (exons 2-12) construct was used as standard [18] (Table S2). For the standard curves, 5 serial 10-fold dilutions from the same reaction mixture of *pUAS-ds* were amplified. For relative quantification, the normalization of different biological samples was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method after efficiency calculation for each primer pair (Table S3). For statistical analysis of the experimental groups, differences in mean values were analyzed with the one-tailed nonparametric Student t-test. A p-value <0.05 was considered statistically significant. Data analysis was carried out using Integromics RealTime StatMiner software (http://www.integromics.com/StatMiner).

Analysis of RACE products

cDNA libraries were obtained with the SMARTer RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech)from two independent biological samples of total RNA of wt imaginal discs and brains. PCR amplification was performed with the Advantage 2 PCR kit (Clontech). Each RACE product was cloned in pCRII vector (Invitrogene), and at least 5 independent clones were sequenced. To confirm RACE results, RT-PCR products were amplified using the Expand High Fidelity PCR System (Roche). The location of primer sequences within the exons and the complete cDNA sequences of DsEx, Ds1 and DsIntra are shown in Figure S2.

Northern blot analysis

Total RNA was extracted from brain and imaginal discs of third instar larvae using TriPure Isolation Reagent (Roche). RNA was quantified in a nanodrop ND-1100. The quality of each sample was checked in an Agilent 2100 bioanalyzer. For Northern blot analysis, 20 µg of total RNA were loaded on 1% agarose-formaldehyde gels and run in MOPS1X buffer. After RNA transfer, the nylon membranes (Roche) were hybridized at 60ºC in modified Church & Gilbert's hybridization buffer [23]. Detection was performed with CDP-Star according to the DIG RNA Detection Protocol (Roche). The digoxigenin-labeled anti-sense RNA probes were synthesized according to the same protocol. As templates, we used two different cDNA fragments: one of 5.2 kb (exons 2-11; clone 119 in Clark, 1995) and the 1.4 kb fragment of exon12 corresponding to the coding sequence of the cytoplasmic domain. The size of the bands was determined by extrapolation from a standard curve using RNA molecular size markers.

Western blot analysis

For Western blot analysis, total proteins were isolated from imaginal discs and brain in Ringer´s solution, supplemented with complete protease inhibitor cocktail (Roche). Aliquots of protein extracts (30 µg) from different genotypes were loaded and separated on 4-15% Mini-PROTEAN TGX Precast Gel (BioRad) and transferred onto PVDF membranes (BioRad). For detection of Ds isoforms containing the cytoplasmic region, a guinea pig anti-Ds antibody (1:2000) and an anti-guinea pig HRP conjugated antibody (1:2000) were used. For detection of Ds isoforms containing the extracellular region, a rabbit anti-Ds antibody (1:2000) [20]and ananti-rabbit HRP conjugated antibody (1:2000) were used. Membranes were developed with the ECL reagent kit.

RESULTS

Expression of Dachsous protein in mutant imaginal discs

To analyze how Ds protein is expressed in different *ds* mutant backgrounds, we used two antibodies - Ds^{ex} and Ds^{cyt} - generated against the extracellular and cytoplasmic

region, respectively (Figure 1). To test their specificity we performed analysis of immunostaining and western blotting (Figure 1B, 1C). As expected, in wild-type (wt) flies, Ds expression inwing discs was similar with both antibodies and preferentially located at the apical membrane of disc proper cells(Figure 1D, 1D'').However, in Western blot the pattern of bands detected by Ds^{ex} and Ds^{cyt} antibodies was different (Figure 1C). Moreover, the bands would correspond to proteins smaller than the reported full-length Ds protein (DsFL; 385 kDa)[17]. Strikingly, Ds^{cyt} antibody revealed a doublet around 50 kDa, not detected by Ds^{ex}.The similar levels of these bands in DsFL and wt cells rule out that they could derive from proteolytic processing of DsFL. Although the results obtained with Ds^{ex} antibody are less conclusive, DsFL seems to be expressed at low levelsin wt cells at larval stage.In contrast, the protein detected by each antibody was different in mutant backgrounds. In ds^{38K}/ds^{36D} discs, Ds was almost undetectable with Ds^{cyt}, whereas the protein detected by $Ds^{ex}$was evenly distributed (Figure 1E', 1E''). Those differences became more evident in ds^{1}/ds^{1}wing discs, in which Ds expression monitored by Ds^{cyt}looked apparently normal, while Ds^{ex}revealed a speckled pattern of Ds distributed between the cytoplasm and the apical membrane (Figure 1F', 1F''). Furthermore, we observed a correlation between the severity of the wing defects and the lack of apical expression detected by Ds^{cyt}.

Therefore, the distribution of Ds and the different bands detected by each antibody suggest the presence of additional isoforms other than DsFL in the imaginal discs and brain.

Quantitative real-time PCR analysis of *ds* expression

To investigate this hypothesis, we performed a quantitative real-time PCR (qRT-PCR) assay to analyze the expression of individual exons in wt imaginal discs and brain - two tissues in which *ds* is preferentially expressed (Figure 2A; Table S1). The full-length cDNA (DsFL) comprises 12 exons (Genbank: L08811.2) and the sequence was determined from the assembly of overlapping clones derived from two embryonic cDNA libraries [17]. Exon12 encodes the complete cytoplasmic region, the transmembrane domain and a small

part of the extracellular domain. Therefore, we decided to split exon12 into two fragments named 12A and 12B to separately analyze the expression of the cytoplasmic region comprised in 12B (Figure 2A). Absolute quantification of individual exons was determined using *pUAS-dsFL* DNA as standard.

An overall comparison showed significant differences in expression levels among individual exons (Figure 2A; Table S2). The highest level corresponded to 12B, with a 10-fold increase with respect to exon2 (the lowest value). These results point out two main conclusions: firstly, according to the relative expression of different exons, several alternative transcriptional isoforms are expressed at this developmental stage. Secondly, DsFL is expressed at lower levels than other transcripts (comparing exon2 to other exons; Table S2). Moreover, differencein the expression of 12A and 12B suggests the existence of novel isoforms containing the cytoplasmic region different to DsFL (exon12) [17].

Characterization of three novel Ds isoforms

To elucidate the molecular structure of putative novel transcripts, we used two different approaches: Northern blot analysis and RACE assay. Up to 8 prominent bands ranging from about 6.0 kb to 0.7 kb were detected with Northern blot hybridization using two different RNA probes comprising either extracellular or cytoplasmic sequences (Figure 2B). Again, the predicted sizes would correspond to potential cDNAs significantly shorter than DsFL (12.4 kb). Strikingly, some bands were exclusively detected by one of the two probes (Figure 2B; red asterisk). Under these experimental conditions we could not detect the band corresponding to DsFL; however, using alkaline conditions to facilitate the transfer of higher bands, a faint band similar in size to p*UAS-ds* (internal control) was detected, indicating that a larger transcript is expressed at low levels (not shown).

Next, RACE assay was used to identify the novel cDNAs. Although specific RACE primers were designed for most *ds* exons, we could only accurately determine the sequence of three novel isoforms named DsEx, Ds1 and DsIntra (Figure 2C; Figure S2). DsEx cDNA comprises exons 1 to 10 and might correspond to the 6.0 kb band detected by

196 Northern blot. Sequence analysis of the 3'RACE (ex10) product showed the existence of a
197 larger novel exon 10 (exon10B) ending with a stop codon followed by a well-defined
198 polyadenylation signal. This result was confirmed by RT-PCR amplification using a forward
199 primer exon1 and the reverse primer ex10.1 (Figure S2C).
200 Ds1 is a small cDNA of 2.6 kb comprising exon1 and exon2B (new 3' end at position
201 2432 in L08811.2) followed by exon12C, a short exon only containing a long
202 polyadenylation signal (11235-11360nt in L08811.2). Ds1 cDNA was determined by
203 sequence analysis of 5'RACE (ex2) and 3'RACE (ex2) products, and was confirmed by RT-
204 PCR amplification (Figure S2C). Accordingly, a band similar in size was detected with both
205 RNA probes by Northern blot (Figure 2B).
206 Unexpectedly, we identified DsIntra, a cDNA that encodes the whole cytoplasmic
207 domain without the trans-membrane domain. The 5´ and 3' ends were accurately
208 determined by sequencing of 5'RACE (ex12) and 3'RACE (ex12) productsamplified with
209 overlapping primers (Figure S2).The 5' untranslated sequence was followed by an ORF that
210 encodes the whole cytoplasmic domain and corresponds to a novel exon named
211 exon12B(initiator methionine at 10156 in L08811.2; Figure S2). Other RACE products were
212 ruled out after sequencing analysis because their sequences were not consistent being
213 probably incomplete RACE products.
214 Next, we checked by Western blot analysis the expression of these potential Ds
215 proteins (Figure 2D). The double bandaround50 kDa, revealed by Ds^{cyt} antibody,could
216 correspond to the predicted size of the DsIntra protein (Figure 2D; red bracket). However,
217 we could not accurately determine the expression of DsEx (217 KDa) that might correspond
218 to a faint band around 220 kDa (Figure 2D; red asterisk).
219 To summarize, using different experimental approaches we have characterized
220 three novel isoforms: the cytoplasmic protein DsIntra, and the extracellular onesDsEx and
221 Ds1 that comprise 17 and 4 cadherin domains, respectively. Nonetheless,the detection of
222 additional bands by Northern andWestern blot suggests the existence of other
223 uncharacterized isoforms.

Depletion of isoforms comprising exon12B reproduces strong mutant phenotypes

To investigate the functional relevance of Ds isoforms, we examined the wing phenotypes generated by the overexpression of two different double-strandedRNA (dsRNAi):dsRNAi-ex and dsRNAi-cyt target the exon6 (extracellular) and exon12B (cytoplasmic), respectively (Figure 1A). The overexpression ofdsRNAi-cyt in the whole wing (638G4) produces phenotypes similar to those observedin strong *ds* alleles (compare Figures 2E and 1E), whereas the wing defects observed with dsRNAi-exare weaker and resemble those observed in ds^{1} and *30AG4* wings (compare Figure 2E and 1F; Figure S3). Interestingly, the molecular lesions of weak alleles map in exons encoding the extracellular region. The different behaviour of both dsRNAi might be explained by either a lower efficiency of dsRNAi-exor a lesser relevance for Ds activityof the isoforms eliminated.To check the interference ability of these dsRNAi, we analyzed the relative expression of individual exons in two *tubG4*/*UAS-dsRNAi*cellsnormalized to wt expression (Figure 2F; Table S3). In both cases, we observed a general decrease in the expression levels, though not identical for each exon. Surprisingly, the silencing efficiency of dsRNAi-ex was higher than that ofdsRNAi-cyt. In dsRNAi-ex cells, the expression of exons 8 and 10 dropped to 0.25similar to targeted exon6,whereas exons such as exon2 or exon9 had 2 or 3 times higherexpression - supporting the existence of alternative splicing isoforms with different extracellular domain. In dsRNAi-cytcells, the expression of exon12B was higher than in dsRNAi-excells (Figure 2F, 2G; fragment 12S). This apparent discrepancy can be explained by the Northern blot results. The presence of several small bands (< 2.3 kb) detected only by the cytoplasmic probe suggests the expression of transcripts encoding only part of the cytoplasmic region (Figure 2B). The transcripts not targeted bydsRNAi-cytcan be quantified by qRT-PCR. Consistently, exon12B expression was undetectable using anotherprimer pair to amplify the whole cytoplasmic domain (fragment 12L) (Figure 2H).

Thus, we conclude that several *ds* transcriptional isoforms are expressed in imaginal discs and brain. These transcripts would encode proteins comprising different extracellular

252 or cytoplasmic domains. Moreover, the isoforms eliminated by dsRNAi-cytexpression play
253 an important role in the regulation of PCP/patterning and growth.
254
255 **Ds is required for mitochondrial function**
256 Recently, it has been reported a novel role of Ft protein regulatingmitochondria
257 activitythrough a C-terminal proteolytic peptide (Ft^{Mito}) imported into the mitochondrion after
258 cleavage of the large transmembrane protein Ft [24]. Ft^{Mito}regulates oxidative
259 phosphorylation. Above, we have shown the importance of the cytoplasmic domain for Ds
260 activity and the expression of DsIntra isoform during larval development. To investigate
261 whether Ds is also required for mitochondrial function, first we examined the expression of
262 Ds with Ds^{ex} and Ds^{cyt} antibodies in fat body cells using COXIV antibody as specific marker
263 for mitochondria (Figure 3). In wt cells, Ds only detected by Ds^{cyt} antibody colocalizes with
264 COXIV indicating that Ds is localized in the mitochondria (Figure 3A). Conversely, in *ds*
265 mutant cells, COXIV stainingis decreased and the number and size of mitochondria is
266 altered compared to wt cells (Figure 3B).Surprisingly, we observed that Ds isoforms
267 detected by $Ds^{ex}$are now accumulated in the nuclei.
268 Next, we examined mitochondrial activity in *ds* mutant cells, analyzing the
269 accumulation of ROS (reactive oxygen species) in wing discs*as* detected by
270 dihydroethidium (DHE) (Figure 3C). In dsRNAi-cyt cells, DHE staining is drastically
271 increased compared to wt cells. Aconsequence of mitochondrial dysfunction is the
272 activation of JNK signaling induced by ROS [25].Accordingly, in dsRNAi-cyt cells we also
273 observed upregulation of *puckered* (puc) gene, a target and functional read out of
274 JNK signaling and activation of Caspase 3 (Figure 3D, 3E). In contrast, the expression of
275 these targets is unaffected in dsRNAi-ex expressing-cells (Figure 3F,3G).Overexpression of
276 DsIntra in P cells results in a smaller P compartment but does not affect the activity of JNK
277 signalling(Figure 3H).
278 These resultsshowa novel function of Ds in the mitochondria. Ds is required for
279 proper mitochondrial morphology, and the depletion of Ds isoforms containing the

cytoplasmic domain promotes elevated levels of ROS and JNK signaling indicating that Ds modulates the activity of the mitochondrial electron transport machinery.

DsIntra contributes to regulate PCP/patterning and growth

The function of the extracellular and cytoplasmic domains of Ds in growth and PCP/patterning has been approached by miss-expression of truncated forms derived from DsFL [14, 15]. These Ds forms consist of proteins anchored to the cell membrane and lacking either the extracellular or the cytoplasmic domains.

To assess the function of DsEx and DsIntra in the regulation of PCP/patterning and growth, we made two transgenes with a C-terminal tag:DsIntraV5 and DsExGFP. The wing phenotypes caused by the overexpression of these Ds isoforms were compared (Figure 4). Furthermore, we quantified their contribution to PCP/patterning and growth by analyzing two different parameters: the A-PCV distance and the relative wing area. To measure wing area variations the transgenes were overexpressed in P cells (hhG4) (Figure 4D-4M; Figure S1).

The wing phenotype of DsIntra and DsFL was similar, indicating that the cytoplasmic domain is sufficient to reproduce the dominant negative phenotype observed in DsFL wings (Figure 4A, 4B, 4D, 4E, 4I, 4J). Indeed, DsIntra and DsFL wings exhibited abnormal polarity (proximal),were smaller and the cross-veins distance was shorter. In contrast, DsEx overexpression did not cause any obvious alteration, the wings looked apparently normal and the hairs orientation was unaffected (Figure 4C, 4F, 4K).To test the ability of DsIntra and DsEx to rescue growth alterations, both were expressed in the P compartment (hhG4) of ds^{38K}/ds^{36D}flies. Unfortunately, DsIntra and DsFL overexpression, consistent with the dominant negative effect, increased lethality at the early pupal stage (> 95%), and only a few adult flies emerged (unfolded wings with more severe alterations). Conversely, DsEx overexpression increased the viability of adult mutant flies without any obvious wing rescue.

Finally, we analyzedhow DsIntra and DsEx overexpression affects the activity of Hippo pathway by monitoring the expressionof*dally-like* (*dlp*) [8] (Figure 4N-4P).Similar to

DsFL, the expression of DsIntra in the P cells(hhG4) leads to downregulation of Dlp expression, whereas DsEx did not cause any detectable change.We also examined Dlp expression in wing discsoverexpressing dsRNAi-ex and dsRNAi-cyt (Figures 4Q, 4R). In accordance with the growth defects observed in the wing, dsRNAi-cyt caused an upregulation of Dlp in P cells (Figure 4R). Strikingly, in dsRNAi-ex cells Dlpexpression was almost eliminated suggesting an increased Hippo activity (Figure 4Q).

These results show that different Ds isoforms contribute to the regulation of PCP/patterning and growthby modulating the activity of the Hippo pathway. Moreover,the opposite effectin Dlp levels observed indsRNAi-ex and dsRNAi-cyt cells would suggestthat certain Dsisoforms could actrepressingthe Hippo activity.

DISCUSSION

The reported *ds* cDNA was derived from an embryonic library, and encodes a single-pass transmembrane cadherin (DsFL) with a large extracellular domain and a small cytoplasmic domain [17]. DsFL is preferentially localized at the apical membrane [18, 20]. However, reported results such as the upregulation of *dlp* expression within the clone of *ds* mutant cells [8]or the inability of ectoDs expression to reverse the hairs polarity in a *ds* mutant background[15] indicate a cell-autonomous mechanism of Ds difficult to envision through the interaction of Ds extracellular domain with Ft, as proposed in the current model.

In this work, using several molecular techniques, we have detectedthe expression of different*ds* transcriptional isoforms that reveal acomplex regulation of the *ds* gene in larval development. We have characterized three novel transcripts that encode the soluble proteins DsEx, Ds1 and DsIntra. Furthermore, qRT-PCR and Western blot data indicate that DsFL is in fact less abundant than other isoforms expressed at this developmental stage. The detection of additional bands by Northern blot and RACE assays suggest the expression of additional isoforms not yet characterized. In addition, the sequence analysis has revealed the existence of exons not previously described such as exon2B, exon10B, exon12B and exon12C.We confirmed by Western blot the expression of DsIntra protein.

The similar levels observed in wt and DsFL expressing-cells rule out the possibility that DsIntra is a peptide derivedby proteolytic cleavage of DsFLas it wassuggested to explain the existence of small bands detected by Ds^{cyt} antibody [26].

A genetic analysis derived from the expression of different Ds isoforms and two specific RNAi has confirmed the functional relevance of the cytoplasmic domain in the regulation of PCP/patterning and growth. Indeed,there is a clear correlation between the severity of the wing defects and the absence of isoforms comprising the cytoplasmic domain monitored at protein (Ds^{cyt}) and RNA (qRT-PCR) levels.Consistently, two proteins that control the Hippo activity such as Riquiqui and Minibrain interact physically with Ds cytoplasmic domain [27]. Although we cannot assign specific functions to individual isoforms, the existence of DsIntra can help to understand the cell-autonomous regulation by Ds of PCP/patterning and growth.However, the new role of Ds in mitochondrial activity further supports the importance of the cytoplasmic domain in the mechanism of action of Ds. Furthermore, the wings phenotypes observed in $ds^{1}$and dsRNAi-ex fliesindicate that isoforms comprising an extracellular domain also participate in the regulation of growth. Interestingly, the expression of Dlp in dsRNAi-excellswould suggest that some isoforms couldnegatively modulate Hippo activity.

In vertebrates, distinct transcriptional isoforms of cadherin-related proteins such as CDH23 and PCDH15 are expressed in different tissues. Also, alternative splice variants of FAT1 have been reported in mouse although the complete cDNAs have not been determined [28]. Therefore, since all these protein are main components of the PCPsignaling cascade [29, 30],this suggest that the existence of isoforms might be anevolutionary conserved mechanism used by these genes toprovidedifferent cellular responses during development [31].

Given that *ds* expression pattern is very dynamic along development, the next challenge will be to determine how its isoforms are spatial- and temporally regulated, in orderto understand how Ds activity can regulate processes such as PCP, patterning, cell

proliferation, mitochondrial activity or even other still unknown.The combination of Ds isoforms in each developmental context could determine the cellular response.

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FIGURE LEGENDS

Figure 1. **Distribution of Ds Protein in different mutant backgrounds**.

A) Schematic drawing of the *dachsous* gene shows the molecular lesion and location of *ds* alleles (red). The exons (blue boxes) correspond to the cDNA that encodes DsFL protein,composed ofan extracellular region with 27 cadherin domains (brown circles), a transmembrane domain (TM; black) and a small cytoplasmic region (cyt; blue line). B) Expression of Ds protein in imaginal wing discs to show the specificity of Ds^{ex} and Ds^{cyt} antibodies. DsFL protein is detected in the P compartment by both antibodies whereas ectoDs form is only detected by Ds^{ex} antibody.A clone of *ds* mutant cells is marked by the absence of GFP.C)Western blot analyses of cell lysates from wt, tubG4>UAS-ectoDs and tubG4>UAS-DsFL imaginal discs and brains. A double-band around 50 kDdetected with Ds^{cyt} antibody is expressed at the same level in DsFL and wt cells (red bracket);howeverthe lower band is eliminated in ectoDs cells suggesting a potential crossregulation between isoforms. An extra band around 40 kD is detected when ectoDs and DsFL are overexpressed.D-F) Adult wings and higher-magnification of the wing proximal region of D) wild type (wt), E) ds^{38k}/ds^{36D} and F) ds^{1}/ds^{1} flies. The impairment of Ds activity can be visualized as smaller and rounded wings, defective vein pattern and a swirling pattern of hairs. XY view and Z-section (white line) of wing discs stained with Ds^{ex} (green) and Ds^{cyt} (red) antibodies. D'') In wt cells, Ds protein is preferentially detected at the apical membrane. E'') Inds^{38k}/ds^{36D} discs (strong alleles), Ds is delocalized and evenly distributed monitored byDs^{ex} antibody and almost undetectable with Ds^{cyt}. F'') Inds^{1}/ds^{1} discs (weak allele), note the differentdistribution of Ds protein detected with Ds^{ex} and Ds^{cyt}. Cells were visualized by Phalloidin staining (Pha). Abbreviations: A anterior ; P posterior

Figure 2. Characterization of novel Ds isoforms. A)qRT-PCR analysis showed high variability in the expression of individual exons in wt cells. 12B: cDNA fragment encoding the cytoplasmic domain. B) Northern blot analysis of total RNA from wild-type cells revealed by two RNA probes. Bands detected only by one of the probes are indicated (red

asterisk).C) Schematic illustration of three novel Ds isoforms. The cDNA and the predicted proteins are shown for each isoforms. Brown boxes: novel exons described in this work. D) Cell lysates from tubG4>UAS-DsIntra cells indicate that the double-band ~50kD would correspond to the DsIntra isoform. E)Adult wings of flies expressing two different dsRNAi in the entire wing (638G4). dsRNAi-cyt wings resemble defects observed in strong *ds* alleles. dsRNAi-ex expression causes mild defects. (F-H) qRT-PCR analysis of individual exons in tubG4>UAS-dsRNAi-ex and tubG4>UAS-dsRNAi-cyt cells. The interference ability of dsRNAi-ex is higher than that of dsRNAi-cyt. (G, H) Exon12B expression in dsRNAi-cyt cells using two different primerpair (12S and 12L: PCR fragments amplified). dsRNAi-cyt expression eliminates the isoforms comprising the whole cytoplasmic domain. Bars show the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3.Ds control mitochondrial activity in the larval fat body.

(A,B)Ds expression infat body cells of third instar larvae. The mitochondria and nuclei were visualized with COXIV (green spots) and DAPI (pink) respectively. A) In wt cells Ds^{cyt} staining is prominent in the mitochondria marked with COXIV antibody. Dsex staining is not detected. B) In ds^{38K} the fat body cellmorphology is altered and the size and number of mitochondria is reduced. Strikingly, in these mutant cells Ds expression is almost undetectable with Ds^{cyt} and Ds^{ex}staining colocalizes with DAPI indicating the abnormalexpression of Ds isoforms with extracellular domain in the nuclei. C) In enG4>UASdsRNAi-cyt wing discs, the mutant territory (P cells) shows elevated ROS levels compared to wt cells (A cells) detected by DHE staining.(D-H) Activity of JNK pathway in enG4>UASdsRNAi-ex, enG4>UASdsRNAi-cytand enG4>UASDsIntra can be observed by monitoring two downstream targets such as pucZ and Cas3. Depletion of Ds isoforms containing the cytoplasmic domain (dsRNAi-cyt) results in a clear upregulation of *pucZ* expression and increased cell-death detected with Cas3. However, neitherthe expression of dsRNAi-ex nor DsIntra caused any change in the expression of these targets. The

514 expression of pucZ and Cas3are shown as an independent panel in grey.Abbreviations: A
515 anterior ; P posterior
516
517 **Figure 4**. **Contribution of different Ds isoforms to the control of PCP/Patterning and**
518 **growth.** Adult wings of flies overexpressing the following transgenes:(A,D) UAS-DsFL,
519 (B,E) UAS-DsIntraV5 and (C,F) UAS-DsExGFP in the entire wing (638G4) and the P
520 compartment (hhG4). (G,H) Adult wings of flies overexpressing dsRNAiex and dsRNAicyt in
521 P cells (hhG4).(I-M) Average of A-PCV distance and wing area of flies expressing DsFL,
522 DsIntra, DsEx, dsRNAcyt and dsRNAiex under the control of 638G4 and hhG4 respectively
523 and normalized to the value of control females. Bars show the mean ± SD. * p< 0.05; ** p<
524 0.01; *** p< 0.001, ns: not significant. (N-R) Analysis of Hippo pathway activity in wing
525 imaginal discs expressing different transgenes in the P cells (hhG4) was monitored by the
526 expression of Dlp. (Q) In wing discs, the overexpression of dRNAi-cyt produced an
527 enlarged mutant territory, whereas (R) in dsRNAi-ex wing discs P compartment was
528 significantly smaller respect to A compartment. The expression of Dlp (grey) is shown as an
529 independent panel.
530
531 **AUTHOR CONTRIBUTIONS**
532 E.R.-Y. performed the qRT-PCR and genetic experiments and analyzed the data. L.V.
533 performed RNA experiments, conducted the confocal imaging and generated Ds antibody.
534 J.S. performed protein experiments and analyzed data from the sequencing and RACE
535 assays. I.R. designed and supervised experiments, analyzed data and wrote the paper. All
536 authors discussed the results and contributed extensively to the work presented in this
537 paper. All authors gave final approval for publication.
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DATA ACCESSIBILITY

The datasets supporting this article have been uploaded as part of the Supplementary Material.

COMPETING INTERESTS

The authors have no competing interests.

Figure 1

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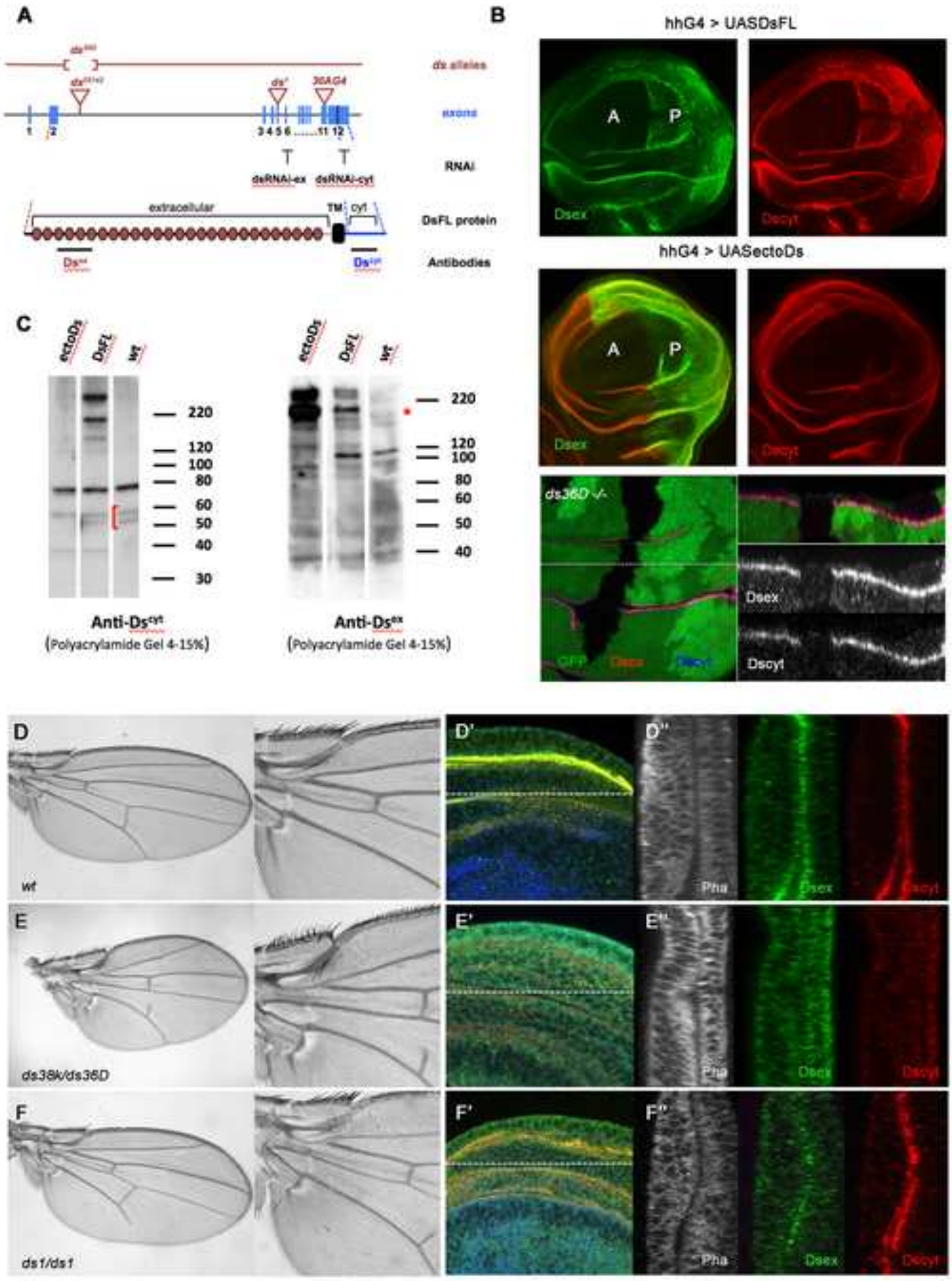

FIGURE 1

Figure 2

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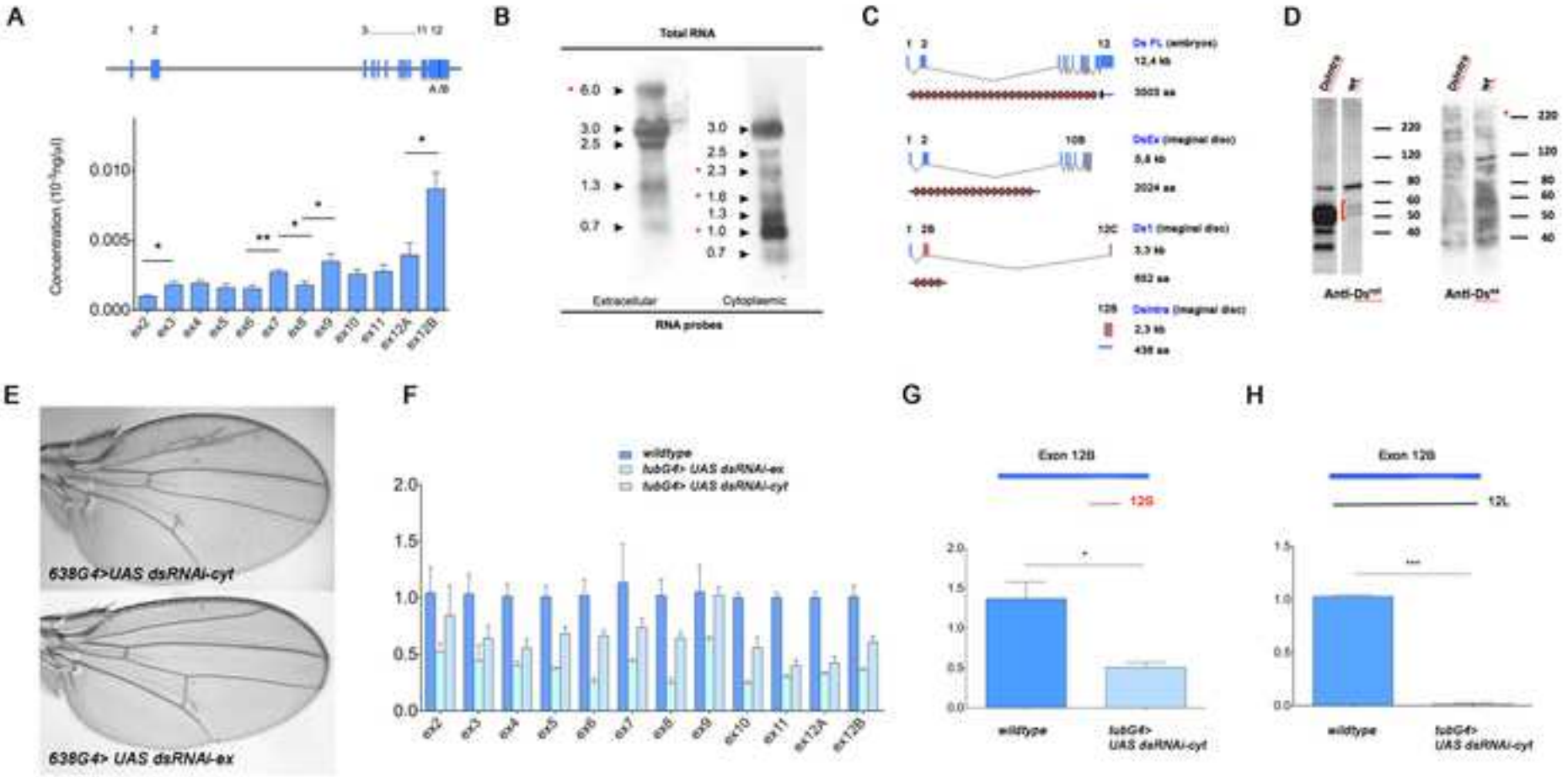

FIGURE 2

Figure 3

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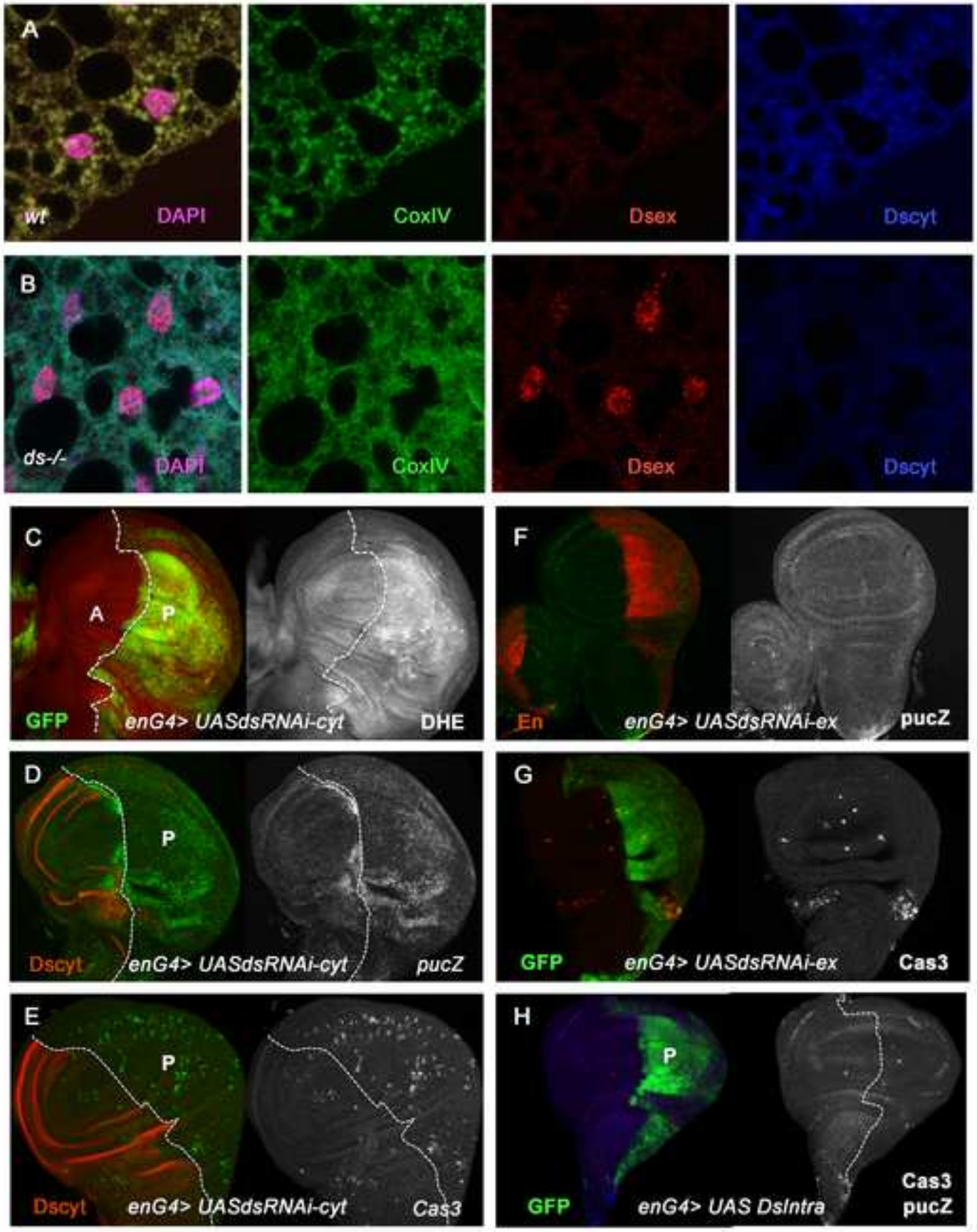

FIGURE 3

Figure 4

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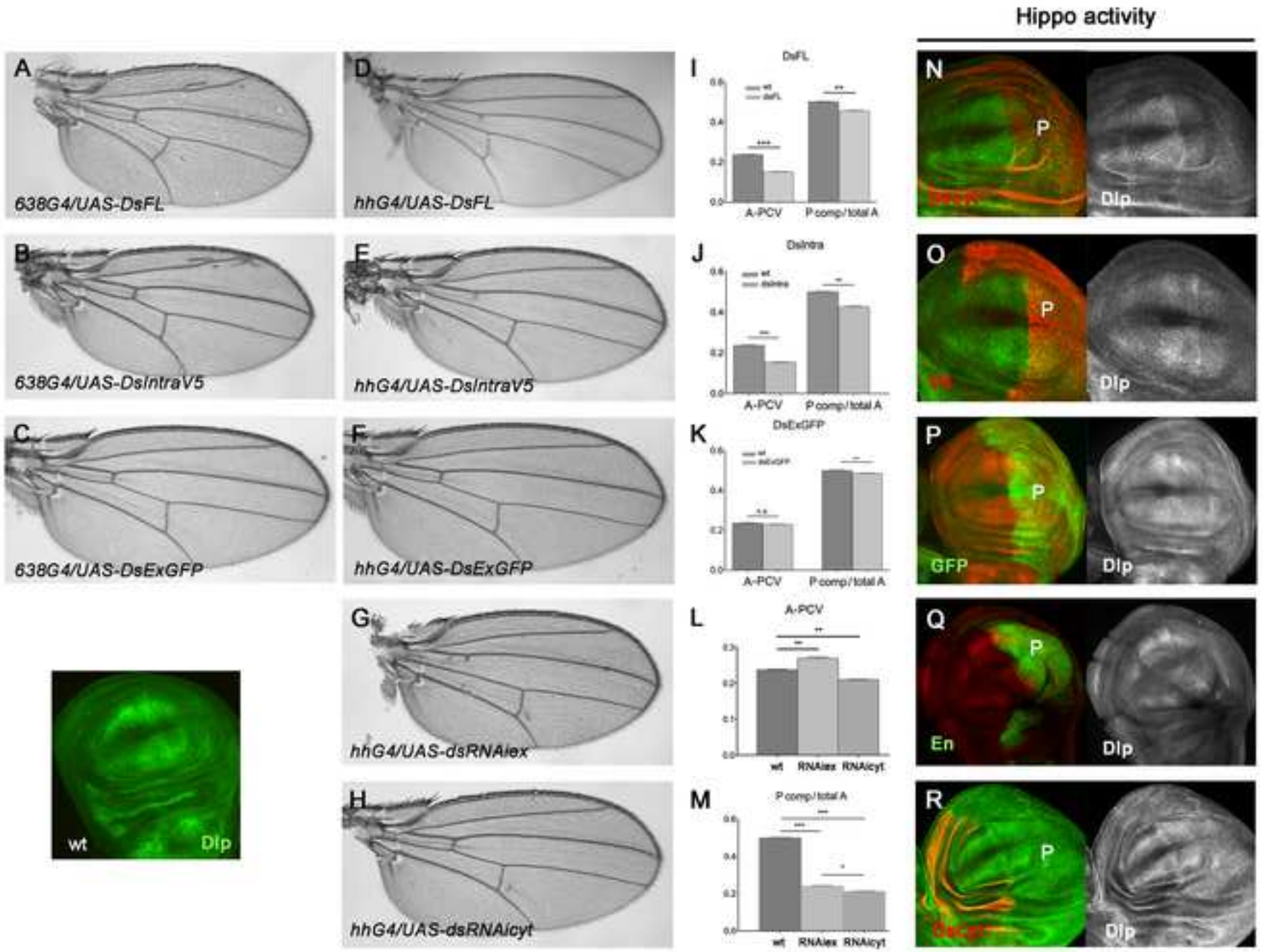

FIGURE 4

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AUTHOR CONTRIBUTIONS

E.R.-Y. performed the qRT-PCR and genetic experiments and analyzed the data. L.V. performed RNA experiments, conducted the confocal imaging and generated Ds antibody. J.S. performed protein experiments and analyzed data from the sequencing and RACE assays. I.R. designed and supervised experiments, analyzed data and wrote the paper. All authors discussed the results and contributed extensively to the work presented in this paper. All authors gave final approval for publication.